

Electronic Supporting Information (ESI) for

Impact of Storage Conditions and Storage Time on Silver Nanoparticles' Physicochemical Properties and Implications for their Biological Effects

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Methods

Nanoparticle preparation

The procedure was as follows: 0.5 mL of 0.1 M AgNO₃ was added to 39.5 mL of capping agent (Na₃C₆H₅O₇ - 0.025%; SDS - 0.05%, chitosan - 0.05% in 0.1% acetic acid solution; BYK-9076® - 0.025% in 0.05% acetic acid solution; Tween 80® - 0.1%; Disperbyk-192® - 0.05%) and stirred at RT. Subsequently, 10 mL of fresh 0.01 M NaBH₄ solution was added dropwise at a speed of 20 mL/h under stirring at RT.

Nanoparticle characterization

The NPs' sizes were determined by DLS using a He-Ne laser (673 nm). Measurements of the stock dispersions were performed in 10 mm polystyrene cuvettes at 25°C. The ZetaPALS Particle Sizing Software ver. 4.10 was used to calculate the particle size. The results are shown as number size distribution to minimize contributions from a small number of larger agglomerates. The zeta potential values were determined using phase analysis light scattering (PALS) Zeta Potential Analyzer ver. 3.29 and calculated using the Smoluchowski model ¹. All measurements were performed five times per batch of NPs.

For the UV-Vis analysis, µL of each solution was added into 3 mL of Milli-Q water and measured across the wavelength range 250-700 nm, which includes the maximum of absorbance (393-462 nm) of AgNPs (5-100 nm).

For TEM analysis, 5 µL of stock dispersions were pipetted onto cobalt grids covered with polyvinyl formal/carbon (S162, Plano GmbH, Wetzlar, Germany) and subsequently left to evaporate. Around 700 NPs were selected to estimate particle size/size distribution using the analySiS pro software (Olympus, Shinjuku, Tokyo, Japan).

The XPS was equipped with a monochromated Al Kα X-ray beam (1486.7 eV) scanned over an area of 600 µm x 400 µm (200 µm diameter/50 W X-ray beam) at a fixed take-off angle of 45°. The pressure in the sample chamber was maintained below 10⁻⁹ mbar. The spectra were collected using 187.5 eV pass energy and 1.0 eV/step. The analyzer slit width was 1.5 nm. For XPS analysis, the stock suspensions were dried onto an indium surface.

MultiPack ver. 9.2 software (Physical Electronics) was used to determine the concentrations of elements and their chemical bonds via line shape analyses.

The XRD was operating at a voltage of 40 kV and a current of 40 mA with Cu K α (1.5406 Å) and K β (1.5444 Å) radiation. For XRD analysis, the stock suspensions were dried onto a silicon surface.

For the determination of crystallite size the software PANalytical HighScore Plus ver.3 was used:

-Fit-Function: Pseudo-Voigt

-Shape-Factor K=0.9

-Reflex, 2 θ

- (1 1 1):38.117; (2 0 0):44.279; (2 2 0):64.428; (3 1 1):77.475

The average crystalline size (d) of all Ag NPs was calculated using Debye-Scherrer formula ².

$$\tau = K \lambda / \beta \cos \theta$$

where:

K is dimensionless shape factor

λ is the wavelength of X-rays

θ is the Bragg diffraction angle

and β is the full width at half-maximum (FWHM) of the diffraction peak corresponding to plane (111).

For all DSC measurements, 8-24% sucrose-in-water gradient was used, and the rotation speed was 20,000 rpm.

100 μ L of particles suspension (100 μ g/mL) was injected after running the appropriate standard suspension.

References

1. M. Smoluchowski: *Annalen der Physik*, 1906, **21**, 756-780.
2. B. D. Cullity: *Elements of X-Ray Diffraction*, Addison-Wesley, Reading, Mass, USA, 3rd edition, 1967.

Changes observed upon suspension in cell culture medium

The behaviour of all batches of NPs in cell culture medium (cMEM) was analyzed using DCS after 6 months of storage. An impact of both NPs' surface capping and storage conditions was observed. Results of DCS measurements are presented in **Table S4**.

Significant differences between the behaviour of NPs capped with different substances were detected, however, these differences did not reflect the changes observed in surface charge. The most stable NPs were those coated with Dysperbyk regardless of the storage conditions, but the second type of neutral AgNPs (Tween) showed significant agglomeration under most storage conditions.

Storage conditions – temperature and access of light – emerged as having a significant impact on the behaviour of studied NPs in cMEM. In most cases, NPs stored at room temperature with access of light displayed very low stability, and in some cases no AgNPs were detected (Ag_Citrate T6-RT-DL). NPs stored at room temperature, but without access of light, showed good stability and generally retained dimensions below 20 nm.

Analysis of silver ions

At storage times T0 and T6, two fractions were prepared for each sample: unextracted aliquots (1 ppb) and extracted aliquots (10 ppb). The unextracted samples were used to quantify the total amount of Ag in the stock solution. The extracted samples were obtained by solid phase extraction (SPE), whereby SPE columns with

sulfonic acid groups (Gracem, Teknolab, Ytre Enebakk, Norway) were used to capture the Ag⁺. All samples were mineralized with concentrated nitric acid (nitric acid ultrapure, Sigma-Aldrich) and analyzed by ICP-MS. The amount of released Ag⁺ was calculated as the difference between the amount of Ag detected in the extracted fractions at T0 and at T6.

Table S1. Stabilizing agents used to protect the Ag NPs from aggregation.

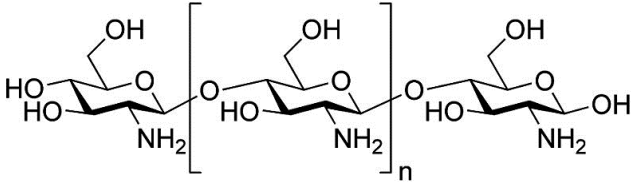
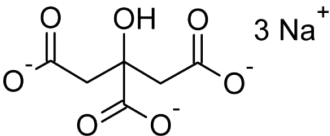
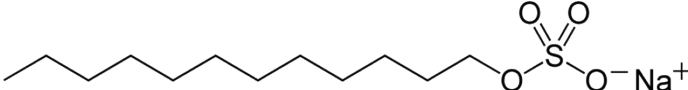
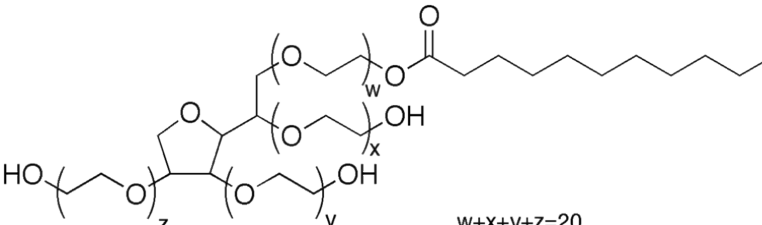
Charge	Name	Molecular weight [g/mol]	Molecular structure
Positive	chitosan	~350000	
	BYK-9076®	-	Alkylammonium salt of a high molecular-weight copolymer
Negative	3-sodium citrate	294	
	sodium dodecyl sulfate (SDS)	288	
Neutral	Tween 80®, Polysorbate 80	1310	
	DISPERBYK-192®	-	Copolymer with pigment affinic groups

Table S2. The average hydrodynamic size of Ag NP and polydispersing index (PDI) analysed by DLS.

Conditions		Ag_Citrate		Ag_SDS		Ag_Disperbyk		Ag_Tween		Ag_Byk	
		Size	PDI	Size	PDI	Size	PDI	Size	PDI	Size	PDI
		[nm]		[nm]		[nm]		[nm]		[nm]	
T0		5.4	0.34	5.0	0.35	9.1	0.23	8.0	0.31	9.2	0.32
T1	4°C-D	5.5	0.33	4.9	0.33	9.3	0.25	8.1	0.30	9.1	0.34
	RT-D	5.7	0.30	5.0	0.30	9.2	0.29	8.5	0.31	9.0	0.34
	RT-DL	6.1	0.33	4.7	0.31	9.3	0.28	9.1	0.32	8.3	0.36
T3	4°C-D	6.9	0.31	5.9	0.29	10.7	0.33	11.2	0.27	22.8	0.30
	RT-D	6.4	0.29	7.3	0.38	10.8	0.35	11.8	0.30	12.5	0.31
	RT-DL	3.1	0.31	13.6	0.23	17.4	0.30	11.6	0.28	26.7	0.29
T6	4°C-D	7.7	0.36	7.7	0.33	12.9	0.31	13.5	0.23	28.1	0.31
	RT-D	6.9	0.36	6.9	0.30	11.7	0.31	9.6	0.27	14.7	0.28
	RT-DL	>1000	-	>1000	-	17.4	0.32	11.6	0.23	24.4	0.30

Table S3. IC₅₀ values (µg/mL) related to an exposure to Ag NPs for RGA and PE assays in the A549 cell line. “x” - IC₅₀ could not be estimated.

IC ₅₀ [µg/mL]						
	Positive		Neutral		Negative	
	Ag_Citrate	Ag_SDS	Ag_Disperbyk	Ag_Tween	Ag_Chitosan	Ag_Byk
RGA						
T0	x	19.46 ±8.79	x	x	5.56 ± 0.27	4.37 ±0.59
T6:4°C	x	20.16 ±0.87	40.73 ±14.45	16.83 ±7.9	4.27 ±1.43	2.98 ± 0.59
T6:RT-D	x	21.31 ±8.15	20.05±5.27	15.65 ±6.63	3.09±0.34	3.26 ±0.1
T6:RT-DL	x	20.54 ±2.89	43.45 ±8.38	x	8.80 ±2.45	3.13 ±0.1
PE						
T0	x	X	x	x	6.72 ±1.83	4.41 ± 0.27
T6:4°C	x	X	x	19.71 ±8.32	2.89 ±0.46	3.74 ±0.23
T6:RT-D	x	23.9 ±5.13	x	14.09 ±8.59	2.08 ±0.51	3.27 ±0.39
T6:RT-DL	x	X	x	x	8.21 ±1.56	3.19 ±0.3

Table S4. DCS measurements: behaviour of AgNPs in cell culture medium after 6 months of storage.

		Size [nm]		
		T6:RT-DL	T6:RT-D	T6:4°C-D
Anionic	Ag_Citrate	-----	15	11 389
	Ag_SDS	21	14	13/12
Neutral	Ag_Tween	356	5	342/349
	Ag_Disperbyk	8	10	9/10
Positive	Ag_Byk	294	20	17/13
	Ag_Chitosan	13	9 35	44/33 316